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Isolation of bacteria from *pulque* with probiotic potential

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ABSTRACT

Objective: To isolate bacteria from *pulque* with probiotic potential for animal feed.

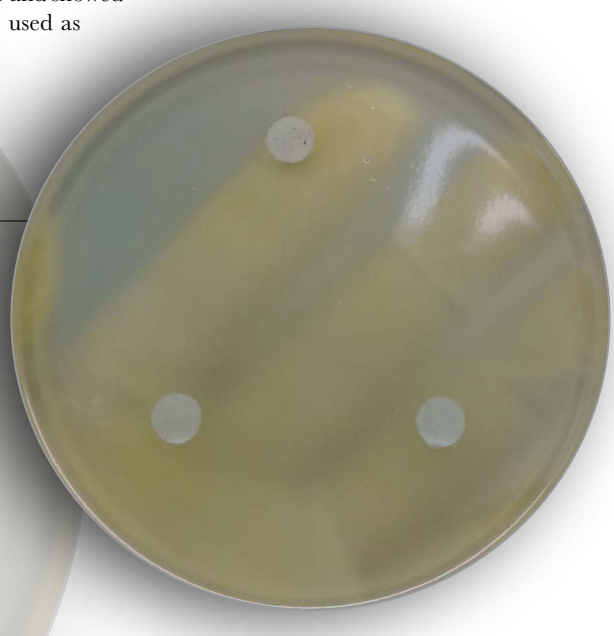
Design/Methodology/Approximation: Samples were taken from *pulque* in the municipalities Tlatlauquitepec, Puebla; Otumba and Tequexquinahuac, Estado de Mexico. For the samples, pH, moisture, ash, and protein were determined. The bacteria colonies were isolated and identified morphologically. Gram dyeing and the catalase test were conducted in pre-selected strains from colonies. In the end, strains with probiotic potential, resistance to pH, biliary salts and antimicrobial activity were identified.

Results: For pH, moisture, ash, and protein in *pulque* samples from Tlatlauquitepec, the results were 3.3, 96.17%, 5.98% and 0.352 g 100 mL^{-1}; from Otumba, 3.25, 97.67% and 0.1763 g 100 mL^{-1}; and from Tequexquinahuac, 2.25, 97.55%, 4.65% and 0.1765 g 100 mL^{-1}. Six different strains were isolated (C2, C3 and C4 in Tequexquinahuac; C5 and C6 in Otumba; and C1 in Tlatlauquitepec). It was found that strain C1 could grow in a pH of 3.0 with survival of 84 % and 73% in biliary salts.

Study Limitations/Implications: Bacteria from *pulque* present probiotic characteristics that can be used for animal feed.

Findings/Conclusions: Strain C1 grew in pH of 3.0 and showed high percentage of survival, which is why it can be used as probiotic in animal feed.

Keywords: Viscous alcoholic beverage, fermentation of agave nectar, intestinal microbiota, pathogenic microorganisms.



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INTRODUCTION

Pulque is a viscous, non-distilled, alcoholic beverage that is produced and consumed in Mexico, product of the fermentation of Agave nectar (Escalante *et al.*, 2008). The fermentation is produced in non-aseptic conditions, which favors the presence of microorganisms (*Kluyveromyces*, *Zymomona*, *Leuconostoc* and *Lactobacillus*) which are found in fermented products with probiotic potential (FAO-WHO, 2006; Valadez-Blanco *et al.*, 2012). Probiotics are an alternative to reduce the use of antibiotics because they are innocuous additives constituted by live microorganisms that improve immune-modulating properties (Angmo *et al.*, 2016). For this, they must be able to survive the passage through the digestive tract, and to resist the action of gastric juices and of biliary salts at the start of the duodenum (Hernández-García *et al.*, 2019). In the search for strains that can be used as probiotics, fermented foods such as pulque are an option, where species that belong to the genera *Leuconostoc* and *Lactobacillus* perform viscous and lactic acid fermentation, while the species *Zymomonas mobilis* and *Saccharomyces cerevisiae* perform alcoholic fermentation (Escalante *et al.*, 2008). Taking into consideration that *pulque* contains a consortium of microorganisms, it can be considered an important source of probiotics; therefore, the objective of this study was to isolate bacteria with probiotic potential from *pulque*, which can be used for animal feed.

MATERIALS AND METHODS

Origin of *pulque* and sampling

In this study samples were taken in three municipalities that produce *pulque* in central Mexico: 1) Ocotlan, Tlatlauquitepec, Puebla (19° 51' 05" LN and 97° 29' 46" LW), 2) Tequexquinahuac (19° 30' 00" LN and 98° 53' 00" LW) and 3) and Otumba (19° 42' 55" LN and 98° 49 '00" LW) in Estado de Mexico (INEGI, 2009). In each site, 500 mL of *pulque* were sampled. The samples were deposited in sterile containers and conserved in ice during their transport to the Animal Nutrition laboratory, Colegio de Posgraduados, Campus Montecillo, where the proximal chemical analysis was performed.

Proximal chemical analysis: pH, moisture, ash and protein

The pH was determined with a potentiometer (Hanna HI 98107) in 100 mL of *pulque*. Moisture and ash were determined by the freeze-drying method, techniques described in AOAC (1995). The determination of total nitrogen was carried out by the micro Kjeldahl method, using 0.5 mL of *pulque* and 2 mL of concentrated H_2SO_4; it was catalyzed and digested until it had an emerald green coloration. It was cooled and transferred to a distiller tube where 30 mL of NaOH at 40% were added. Later, an Erlenmeyer flask with 6 mL of H_3BO_3 at 4% with indicator was placed and the distiller was turned on. Finally, titration of the distillate was conducted with HCl 0.05 N.

Isolation and morphologic identification of colonies

Inside a gas extracting hood (LABCONCO class II type A2), serialized dilutions were carried out (101 to 106) using 9 mL of saline solution and 1 mL of the *pulque* sample, and aliquots of 0.5 mL from each dilution were sown by surface extension in Petri dishes

with Man-Rogosa-Sharpe-Agar (MRS) medium, methodology described by Cervantes-Contreras and Pedroza-Rodríguez (2007).

Pre-selection of strains: Gram dyeing and catalase test

Catalase-negative colonies and Gram-positive bacillus were selected. Gram dyeing was made taking a sample from the isolated colony and deposited in a slide together with a drop of distilled water; it was left to dry, and the smear was fixed by heat (Harrigan, 1976). Selection of strains with probiotic potential: resistance to pH, biliary salts, and antimicrobial activity

After the biochemical tests, strains were selected with characteristics that define probiotics such as survival to the digestion process, which is why the strains selected were subjected to tests such as resistance to acid pH, biliary salts and their action described by Gómez-Zavaglia *et al.* (1998) and Da Silva-Ferrari *et al.* (2016).

RESULTS AND DISCUSSION

Physicochemical characteristics

In this study the pH ranged from 2.55 to 3.30 (Table 1), values that are below the limits of quality included in the Mexican norm NMX-V-037-1972 (Secretaría de Economía, 1972), which range from 3.5 to 4.0. With higher fermentation there is an increase in the production of lactic acid from lactic acid bacteria as in the production of ethanol through microorganisms of genera *Zymomonas* and *Saccharomyces* (alcoholic fermentation), reducing the sugar content and acidifying the medium (Escalante *et al.*, 2008). This is an ideal characteristic for pulque, since it is given by different microorganisms that give rise to the three fermentative processes: lactic, alcoholic, and viscous (Lappe-Oliveras *et al.*, 2008).

For its part, moisture was similar between the three samples evaluated, with ranges of 96.17 to 97.67% (Table 1); this was parallel to what was reported by Anderson *et al.* (2009), who found 98% of moisture in pulque samples. Regarding ash, 5.98% and 4.65% were found in this study in Tlatlauquitepec and Tequexquinahuac, respectively, which were results higher than those found by other studies that report lower contents (from 0.3 to 0.5%). These results are because of the way that the Agave nectar extraction is performed, since a large amount of solids with the minerals that could be in the form of ash in *pulque* are separated when this is done (Escalante *et al.*, 2016).

Finally, 0.352, 0.1763 and 0.1765 g of protein 100 mL^{-1} were obtained in Tlatlauquitepec, Otumba and Tequexquinahuac (Table 1). Results of 0.14 to 0.31 g protein 100 mL^{-1} were found by León-de la O *et al.* (2012), while Anderson *et al.* (2009) reported 0.6 g protein 100 mL^{-1}. The concentrations of protein and pH in *pulque* are quite variable,

Table 1. Proximal chemical analysis of *pulque* samples from the three municipalities sampled.

Samples	Hp	Humidity (%)	Ash (%)	Protein (g 100 mL^{-1})
Tlatlauquitepec	3.30	96.17	5.98	0.352
Otumba	3.25	97.55	4.10	0.1765
Tequexquinahuac	2.55	97.67	4.65	0.1763

since they are determined by the time of fermentation (Escalante *et al*., 2008). Values of 0.37 g protein 100 mL^{-1} have been reported at the beginning of the nectar's fermentation; 48 h later, the concentration decreases to 0.10 g protein 100 mL^{-1} (Cervantes-Contreras and Pedroza-Rodríguez, 2007). Therefore, the characterization of *pulque* is complex since it involves factors that intervene in its production: microorganisms, type of maguey and of soil, climate, time of harvest, and innocuousness practices that were conducted during the process (Angmo *et al*., 2016).

Isolation and morphological identification of colonies

Six strains were isolated in the study (C); C2, C3 and C4 correspond to Tequexquinahuac, C5 and C6 to Otumba; and C1 to Tlatlauquitepec (Table 2). The strains C2, C3, C4 and C5 have morphological characteristics associated to yeasts. The characteristics found in this study agree with what was reported by Escalante *et al*. (2016): white, creamy colonies, large and with regular edges. The strains C1 and C6 presented morphological characteristics of *Lactobacillus* spp. (Figure 1a), which were reported in studies carried out by León-de la O *et al*. (2012).

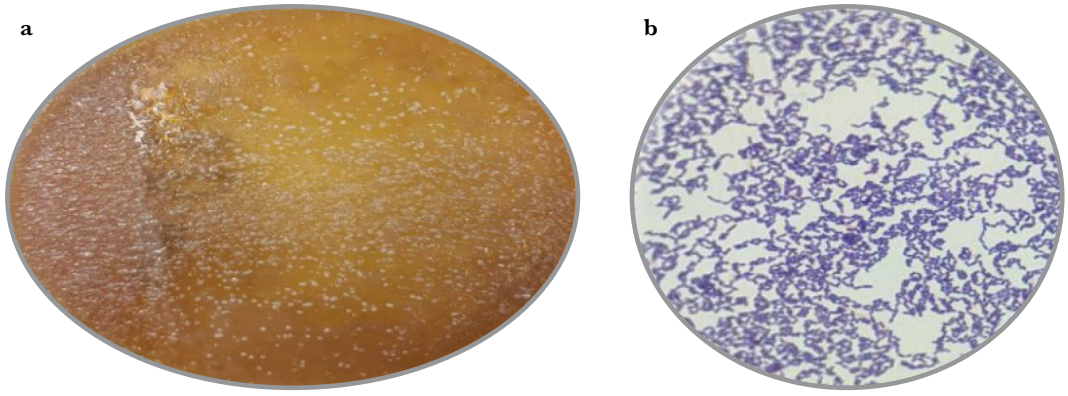

Figure 1. Strain C1 isolated from the sample from Tlatlauquitepec, Puebla (a), and its Gram dyeing (b).

Table 2. Morphological characteristics of the colonies isolated from *pulque* collected in three municipalities.

Characteristic	Tlatlauquitepec	Tequexquinahuac			Otumba	
	C1	C2	C3	C4	C5	C6
Size	Small	Medium	Large	Medium	Large	Small
Colour	White	White	Cream	White	White	Cream
Form	Round	Concave	Round	Round	Round	Round
Elevation	Convex	Convex	Flat	Convex	Convex	Flat
Area	Lisa	Lisa	Lisa	With reliefs	Lisa	Lisa
Borders	Continuos	Continuos	Continuos	Irregulares	Continuos	Continuos
Reflected light	Bright	Bright	Opaque	Bright	Bright	Opaque
Appearance	Creamy	Creamy	Creamy	Secas	Creamy	Creamy

When performing Gram dyeing and the catalase test in the six isolated strains, only C1 fulfilled the characteristics that distinguish lactic acid bacteria of the genus *Lactobacillus*, which are: positive Gram dyeing, bacillus shape and negative catalase (Table 3; Figure 1b). Therefore, it was the only one selected for the probiotic potential tests.

For a microorganism to be considered probiotic, it must be viable at the moment of consumption. Although there is still not an adequate dose, the food industry and the Food and Drug Administration (FDA) recommend an amount of 10-6 CFU mL^{-1} (Tripathi and Giri, 2014). The CFU of each dilution corresponds to each strain obtained presented in Table 4; as can be seen, C1 from Tlatlauquitepec was the one that presented a higher concentration (2×10^{-6} CFU mL^{-1}) which is within the recommendations to be supplied as probiotic. In its part, C4 was the one that obtained the lowest recount. The CFU mL^{-1} values obtained in the samples analyzed are low compared to what was reported by Escalante *et al.* (2008) who describe that lactic acid bacteria predominate during the first hours of fermentation of the Agave nectar with pulque (BAL; 1.5×10^{-8} CFU mL^{-1}), followed by aerobic mesophilic bacteria (1.2×10^{-7} CFU mL^{-1}) and yeasts (8.8×10^{-6} CFU mL^{-1}), stating as so until the end of the fermentation.

Probiotic potential tests

The C1 strain isolated from Tlatlauquitepec grew with pH of 3.0 and showed a survival of 84% (Table 5). Similar data were found by González-Vázquez *et al.* (2015) when they subjected the sample isolated from *pulque* to a pH of 1.5 for 4 h and found survival of

Table 3. Gram dyeing and catalase test of the isolated strains.

Samples	Strains	Gram stain	Form	Catalase Test
Tlatlauquitepec	1	Positive	Bacilli	Negative
Tequexquinahuac	2	Positive	Leaven	Positive
	3	Positive	Leaven	Positive
	4	Positive	Leaven	Positive
Otumba	5	Negative	Bacilli	Positive
	6	Positive	Bacilli	Positive

Table 4. Counting of colony forming units (CFU mL^{-1}) per municipality sampled.

Samples	Strains	Dilutions					
		10^1	10^2	10^3	10^4	10^5	10^6
Tlatlauquitepec	M1	UCT	UCT	232	60	12	2
Tequexquinahuac	M2	UCT	UCT	UCT	646	130	-
	M3	UCT	UCT	UCT	16	-	-
	M4	134	-	-	-	-	-
Otumba	M5	UCT	98	20	4	-	-
	M6	UCT	232	-	-	-	-

UNC=Uncountable. The dash means bacteria growth.

Table 5. Percentage of survival of the C1 strain isolated from Tlatlauquitepec.

Dilutions	Control (UFC mL^{-1})	Acidified Hp 3 (UFC mL^{-1})	Survival (%)	Control (UFC mL^{-1})	Bile salts 0.5% (UFC mL^{-1})	Survival (%)
10^1	UCT	UCT	UCT	UCT	UCT	UCT
10^2	UCT	UCT	UCT	UCT	UCT	UCT
10^3	UCT	UCT	UCT	UCT	UCT	UCT
10^4	UCT	UCT	UCT	UCT	UCT	UCT
10^5	UCT	UCT	UCT	UCT	UCT	UCT
10^6	390	380	97.43	310	156	50.32
10^7	80	76	95	92	89	96.73
10^8	13	8	61.53	-	-	-
Mean			84.65	201	122.5	73.53

UNC: Uncountable, CFU: colony forming units (CFU). The dash means without bacteria development.

60% of the *L. casei* strain. For their part, Cervantes-Elizarrarás *et al.* (2019) determined the probiotic properties of 10 BAL strains of the genus *Lactobacillus* isolated from *pulque*, subjecting the strains to a pH of 2 during 2 h, obtaining a survival of 63.2 to 96.3%. Escalante *et al.* (2016) mention that when the tolerance of BAL to a medium with pH of 3.0 is evaluated *in vitro*, it should be done for 3 h, average time that digestion takes. The results obtained indicate that C1 can be a candidate to be used as probiotic (González-Vázquez *et al.*, 2015). The resistance of the strains to conditions of acid pH has been attributed to the different regulation mechanisms of intracellular pH and alkalization of the external environment through decarboxylation and deamination reactions (Tripathi and Giri, 2014).

Biliary salts

The *in vitro* evaluation of the strain selected was conducted at a concentration of 0.5% of biliary salts, incubating it at 37 °C during 24 h. C1 showed a survival of 73% (Table 5). These results are similar to those found by Castro-Rodríguez *et al.* (2015) where four strains of *L. mesenteroides* isolated from pulque had a survival of 88 to 99% subjected to 0.5% of biliary salts. For their part, Cervantes-Elizarrarás *et al.* (2015) obtained different results from those in this study, where three out of the ten strains of BAL isolated from *pulque* showed a resistance to biliary salts (0.5%) of 52.5 to 55.7%, and the remaining strains obtained a resistance of 58.8 to 66%. The capacity for resistance to biliary salts is attributed primarily to the presence of the hydrolase enzyme of biliary salts, which is secreted by some lactic acid bacteria of the genus *Lactobacillus* (Begley *et al.*, 2006). The results obtained both in the pH test and in that of biliary salts indicate that the C1 strain is a candidate to be probiotic. A probiotic strain is capable of surviving as it passes through the stomach (pH=3.0) and small intestine (biliary salts 0.5%) (Hernández-Mancipe *et al.*, 2019), places where these two natural and important barriers are found in mammals.

Antimicrobial activity against pathogenic bacteria

The C1 strain did not show antimicrobial activity against the pathogenic bacteria *E. coli* ATCC 25922 and *Salmonella typhimutium* ATCC 14028 (Figure 2). In a study carried out by Cervantes-Elizarrarás *et al.* (2019), the authors found that 4 out of 10 strains of BAL isolated from pulque did not present antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*. There are reports that lactic acid bacteria and *Leuconostoc mesenteroides* P45 isolated from pulque have activity against enteropathogenic bacteria *E. coli* (EPEC), *L. monocytogenes*, *S. enterica* serovar *Typhi* and *Typhimuriu* (Giles-Gómez *et al.*, 2016). The lack of antimicrobial activity presented by the C1 strain can be attributed to the absence of conditions for anaerobiosis.

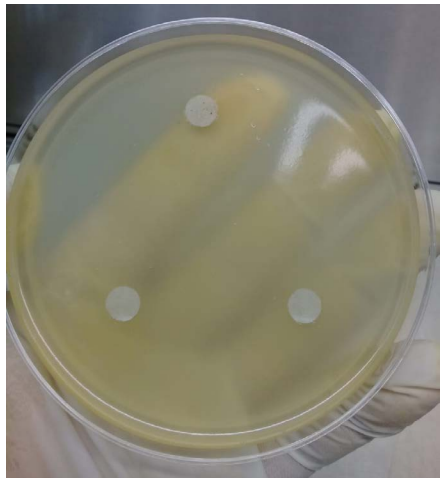

Figure 2. Results from the antimicrobial activity of strain C1.

CONCLUSIONS

It was found that the strain from Tlatlauquitepec, Puebla (C1), grew in pH of 3.0 and showed a high percentage of survival; therefore, it can be used as probiotic in animal feed. However, it did not show antimicrobial activity against pathogenic bacteria *E. coli* ATCC 25922 and *Salmonella typhimutium* ATCC 14028.

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